Characterization of the Expression of TLR2 (Toll-like Receptor 2) and TLR4 on Circulating Monocytes in Coronary Artery Disease

Kazuhiro Ashida¹, Koji Miyazaki¹, Eiji Takayama², Hironori Tsujimoto³, Makoto Ayaori¹, Tadayuki Yakushiji¹, Noriyuki Iwamoto¹, Astushi Yonemura¹, Kikuo Isoda¹, Hidetaka Mochizuki⁴, Hoshio Hiraide³, Masatoshi Kusuhara¹, and Fumitaka Ohsuzu¹

TLRs are receptors involved in the recognition of pathogens by the innate immune system, and TLR2 and TLR4 play important roles in the activation of monocytes. A total of 105 consecutive patients who underwent coronary angiography comprised of 46 with stable effort angina (SA), 41 with unstable angina (UA), and 18 with no significant CAD (CNT) were enrolled. The baseline expression levels of TLR2 and TLR4 on monocytes in peripheral blood mononuclear cells (PBMCs) were determined by flow-cytometric analysis. Since TLR2 expression has been reported to be regulated by TLR4 signaling, we cultured PBMCs with or without lipopolysaccharide (LPS, 1 µg/ml). At baseline, TLR4 levels (mean of fluorescence intensity) in SA (145 ± 58, p < 0.05) and UA (164 ± 65, p < 0.01) were higher than those in CNT (107 ± 37). As for TLR2, levels were higher in UA (108 ± 36, p < 0.05) than in SA (94 ± 18) and CNT (87 ± 22). After stimulation with LPS, TLR2 levels increased in SA but decreased in UA. In conclusions, TLR4 levels increased in both SA and UA. Monocytes in UA were characterized by elevated TLR2 levels and unresponsiveness of the TLR2 levels to TLR4 stimulation. J Atheroscler Thromb, 2005; 12: 53–60.

Key words: Unstable angina, Stable effort angina, Monocyte, Lipopolysaccharide

Introduction

Atherosclerosis is now considered to be a chronic inflammatory process due to arterial injury (1–5). Pathological studies have shown that there is invasion of macrophages and T-lymphocytes into atherosclerotic plaques, which suggests that both innate and acquired immunity are involved in the pathogenesis of atherosclerosis (5, 6). Since acquired immunity is induced by innate immunity, it is important to clarify the mechanism for the activation of innate immunity in the pathogenesis of atherosclerosis.

Toll-like receptors (TLRs) have been identified as receptors involved in microbial recognition by the innate immune system (7). TLRs belong to the pattern recognition receptors that recognize the pathogen-associated molecular pattern, common molecular features of target microorganisms. To date, 10 TLRs have been identified (7). When TLRs on macrophages are activated, this leads to activation of the NF-κB pathway which brings about the production of pro-inflammatory cytokines and expression of co-stimulatory molecules, resulting in the induction of acquired immunity (8–10). In the context of atherosclerosis, evidence suggests that TLRs are involved in human atherosclerosis (11, 12). Pathological examination of human atherosclerotic plaques have shown that TLR4, a receptor for the lipopolysaccharide (LPS) of gram negative-bacteria, and TLR2, a receptor for the pepti-
doglycan of gram-positive bacteria, are expressed on macrophages invading atherosclerotic plaques. In this case, the immunoreactivity of these receptors was co-localized with the nuclear translocation of NF-κB, a marker of the activation of this pathway, suggesting that these TLRs could play an important role in macrophage activation in such plaques (11).

Clinical studies have demonstrated the effects of polymorphism of the TLR4 gene on the progression of atherosclerosis (13, 14). In this regard, it has been shown that the Asp299Gly allele of the TLR4 gene, which reduces the effectiveness of signal transduction by this receptor, is associated with slower progression of atherosclerosis in the carotid artery, suggesting that the activation of the TLR4 pathway could play an important role in the progression of atherosclerosis (14).

Atherosclerosis [including that in coronary artery disease (CAD)] has been reported to be associated not only with local inflammation in the arterial walls but also with the systemic inflammatory response (15, 16). In the case of patients with CAD, previous studies have suggested that both stable effort angina (SA) and unstable angina (UA) were associated with a systemic inflammatory response, though to different extents (17).

We speculated that TLR2 and TLR4 are involved in the activation of circulating monocytes in patients with CAD and that their expression levels may therefore change depending on monocyte activation levels. So, we measured the expression levels of TLR2 and TLR4 on circulating monocytes in patients with SA and UA as well in patients with angiographically normal coronary arteries (CNT) and found the monocytes of UA patients to be characterized by increased TLR2 levels. TLR4 levels were elevated to about the same extent in UA and SA and higher than those in CNT.

It has been reported that there is cross talk between TLR4 and TLR2 (18), through which TLR2 expression levels are upregulated by TLR4 stimulation (19–22). In view of this, we also studied changes in TLR2 expression levels in response to TLR4 stimulation induced by LPS. Though there was no difference in TLR4 expression levels between SA and UA patients, the responsiveness of TLR2 to TLR4 stimulation differed between these two patient groups. As expected, TLR2 levels were upregulated by TLR4 stimulation in the SA patients, but interestingly, they were downregulated by TLR4 stimulation in the UA patients.

**Materials and Methods**

**Patient population**

One hundred and five consecutive patients who underwent coronary angiography for suspected ischemic heart disease between January 2002 and February 2003 were enrolled in this study. Patients with advanced kidney or liver disease, heart failure, acute or recent myocardial infarction within 6 months or a history of major surgery or trauma within the previous month were excluded. The study protocol was approved by the Ethics Committee of the National Defense Medical College and written informed consent was obtained from all patients. We divided the patients into 3 groups—SA, UA and CNT—with 46, 41 and 18 patients, respectively, in each group. The SA patients had had no acute events or any worsening of symptoms during the previous 6 months. The diagnosis of UA was made according to the classification of Braunwald (19) and any patients with an elevation in either serum creatinine kinase and/or Troponin T levels at admission were excluded (Table 1).

**Peripheral blood mononuclear cells**

Heparinized venous blood samples (4 ml) were taken at admission. Within 30 minutes of sampling, peripheral blood mononuclear cells (PBMCs) were isolated from the blood samples using Lymphocyte Separation Medium (ICN, Biomedical Inc., Aurora, OH, USA). The expression levels of TLR4 and TLR2 on CD 14 positive PBMCs were determined by flow cytometric analysis at baseline as well as after culturing PBMCs in the presence or absence of LPS.

**Table 1. Clinical Backgrounds of the Three Groups.**

<table>
<thead>
<tr>
<th></th>
<th>CNT (N = 18)</th>
<th>SA (N = 46)</th>
<th>UA (N = 41)</th>
<th>P</th>
</tr>
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<tbody>
<tr>
<td>Age (y)</td>
<td>58 ± 11</td>
<td>66 ± 9</td>
<td>64 ± 11</td>
<td>0.042</td>
</tr>
<tr>
<td>Sex (M/F)</td>
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<td>39 / 7</td>
<td>29 / 12</td>
<td>ns</td>
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<tr>
<td>Risk factors, n (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hypertension</td>
<td>7 (39)</td>
<td>20 (43)</td>
<td>24 (59)</td>
<td>ns</td>
</tr>
<tr>
<td>Smoking</td>
<td>8 (44)</td>
<td>21 (46)</td>
<td>22 (54)</td>
<td>ns</td>
</tr>
<tr>
<td>Hypercholesterolemia</td>
<td>4 (22)</td>
<td>22 (48)</td>
<td>21 (51)</td>
<td>ns</td>
</tr>
<tr>
<td>Diabetes</td>
<td>2 (11)</td>
<td>8 (17)</td>
<td>14 (34)</td>
<td>0.025</td>
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<td>Therapy, n (%)</td>
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<td></td>
</tr>
<tr>
<td>Aspirin</td>
<td>14 (78)</td>
<td>42 (91)</td>
<td>33 (80)</td>
<td>ns</td>
</tr>
<tr>
<td>Beta-blockers</td>
<td>8 (44)</td>
<td>11 (24)</td>
<td>17 (41)</td>
<td>ns</td>
</tr>
<tr>
<td>ACE inhibitors</td>
<td>6 (33)</td>
<td>11 (24)</td>
<td>8 (20)</td>
<td>ns</td>
</tr>
<tr>
<td>Nitrates</td>
<td>6 (33)</td>
<td>20 (43)</td>
<td>11 (27)</td>
<td>ns</td>
</tr>
<tr>
<td>Statins</td>
<td>4 (22)</td>
<td>17 (37)</td>
<td>17 (41)</td>
<td>ns</td>
</tr>
<tr>
<td>Calcium-channel blockers</td>
<td>9 (50)</td>
<td>23 (50)</td>
<td>25 (61)</td>
<td>ns</td>
</tr>
<tr>
<td>Angiographic findings, n (%)</td>
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<td>1-Vessel disease</td>
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<td>26 (57)</td>
<td>18 (44)</td>
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<tr>
<td>2-Vessel disease</td>
<td>0</td>
<td>13 (28)</td>
<td>9 (22)</td>
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<tr>
<td>3-Vessel disease</td>
<td>0</td>
<td>7 (15)</td>
<td>14 (34)</td>
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</tr>
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</table>
**Cell cultures and LPS stimulation**

PBMCs (1 × 10^6 cells / 2 ml / well) were cultured in RPMI-1640 medium containing 10% human serum and antibiotics/antimycotics (Life Technologies, Ground Island, NY, USA) in the presence or absence of 1 µg/ml of LPS (Escherichia coli 127, B8; Sigma-Aldrich Co., St. Louis, MO, USA) for 24 hours in 24-well culture dishes under 5% CO₂.

**Flow cytometric analysis**

Firstly, TLR4 expressed on PBMCs were stained with mouse anti-human TLR4 mAb (HTA 125; Medical & Biological Laboratories Co., Aichi, Japan), and then washed with staining buffer (phosphate-buffered saline (PBS) containing 5% fetal calf serum, 10 mM EDTA and 0.1% sodium azide). Next, development was conducted with Biotin-conjugated rabbit anti-mouse immunoglobulins (Dako, Glostrup, Denmark) and PE-conjugated streptavidin (Medical & Biological Laboratories Co.) and the preparations were washed twice with staining buffer. Secondly, CD14 and TLR2 expressed on PBMCs were stained with APC-conjugated anti-human CD14 mAb (RMO 52; Medical & Biological Laboratories Co.) and FITC-conjugated anti-human TLR2 mAb (TL 2.1; CASCADE, MA, USA), and then preparations were washed with staining buffer. After these procedures, the PBMCs were re-suspended in staining buffer, and placed in a flow cytometric analyzer (FACS Calibur; Becton Dickinson, Cockeysville, MD, USA) Analysis of the fluorescence intensities (MFI) determined for TLR4 and TLR2 on monocytes expressing CD14 was conducted using Cell Quest software (Becton Dickinson).

**Statistics**

All results were expressed as median values (IQR). Differences in patient characteristic parameters were analyzed using Pearson’s χ² test and any significant differences between the 2 groups were evaluated using the Mann-Whitney U test. Values in more than 3 groups were tested by a 1-way analysis of variance (ANOVA) and then applying the Kruskal-Wallis test. A value of p < 0.05 was regarded as significant. All statistical analyses were performed with the SigmaStat software package (SPSS) 6.1 for the Macintosh.

**Results**

**Patient characteristics**

The median age of the SA group was higher than that of the UA CNT groups. In the UA group, there were more diabetic patients than in the other groups and this group had more patients with multi-vessel disease than the SA group. Other than these findings, however, there were no significant differences between the three groups as regards their clinical background, which included coro-

**Baseline levels of TLR2 and TLR4**

Baseline TLR2 levels were significantly higher in the UA group (mean fluorescence intensity; 107.5 ± 36.3) than in the SA (94.0 ± 17.7, p = 0.043) and CNT (86.9 ± 21.6, p = 0.031) groups. They were not significantly different between the SA and CNT groups (Fig. 1).

The baseline TLR4 levels were significantly higher in the SA (145.3 ± 58.2) and UA (163.8 ± 64.5) groups than in the CNT (106.9 ± 36.7) group (SA vs CNT; p = 0.012, UA vs CNT; p = 0.0008). They were not significantly different between the SA and UA groups (Fig. 2).

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**Fig. 1.** Baseline TLR2 expression levels on monocytes in the three groups. The expression levels were measured as the mean of fluorescence intensity and the bar plots show medians in the 25th and 75th percentiles. Kruskal-Wallis test: p = 0.04

**Fig. 2.** Baseline TLR4 expression levels on monocytes in the three groups. Expression levels were measured as the mean of fluorescence intensity and the bar plots show medians in the 25th and 75th percentiles. Kruskal-Wallis test: p = 0.0019
Changes in TLR2 and TLR4 levels in response to LPS stimulation

To quantify the effect of LPS stimulation on the expression levels of the TLRs, the TLR levels of LPS stimulated samples were normalized to those of the corresponding control samples with no LPS stimulation, and levels were expressed as percentages. Figure 3 is a representative example showing the effect of LPS stimulation on the TLR2 and TLR4 levels on monocytes from SA and UA patients. After culturing PBMCs from SA patients with or without LPS, TLR2 levels were 189 and 166, respectively. When normalized, the value for the LPS stimulated sample was 114% meaning that LPS stimulation had increased the TLR2 level by 14%. After culturing PBMCs from UA patients with or without LPS, the TLR2 levels were 208 and 242, respectively. In this case, normalization produced a value of 86% indicating that LPS stimulation had decreased the TLR2 level by 14%. With the same SA and UA patients, LPS stimulation increased TLR4 levels 47 and 1%, respectively.

The effect of LPS stimulation on the TLR2 levels in SA and UA patients is shown in Fig. 4. In the SA group, TLR2 was up-regulated in 42 of the 46 patients (91.3%), while in the UA group TLR2 was down-regulated in 37 of the 41 patients (90.2%). In the SA patients, the TLR2 levels of LPS stimulated samples were significantly higher than those of control samples (114 ± 11% of control values, p < 0.0001) (Fig. 5). In the UA patients, the TLR2 levels of LPS stimulated samples were significantly lower than those of control samples (91 ± 11% of control values, p < 0.0001) (Fig. 6).

The effect of LPS stimulation on the TLR4 levels in the SA and UA patients is shown in Fig. 7. In both the SA and UA group, the response of TLR4 levels to LPS stimulation varied, with a down-regulation in about half of the patients and up-regulation in the other half. TLR4 levels in the LPS stimulated samples were not significantly different from those of control samples for both SA (125 ± 57% of control values) and UA (103 ± 35% of control values) patients. Also, the normalized expression levels

![Fig. 3. Representative cases showing effect of LPS stimulation on TLR levels in SA and UA patients. Expression levels of TLR2 and TLR4 on monocytes from patient with SA or UA after cultivation with or without LPS (1ug/ml) for 24 hours. Solid lines and dotted lines indicate the distribution of fluorescence intensity in LPS stimulated and unstimulated monocytes, respectively. The expression levels are indicated as MFI (mean of fluorescence intensity).](image-url)
Toll-like Receptors in Coronary Artery Disease

Monocyte-macrophages have been reported to play a key role in the pathogenesis of atherosclerosis and microorganisms such as Chlamydia pneumoniae and cytomegalovirus have been found to aggravate atherosclerosis by activating immune systems in the host. Further, toll-like receptors have been shown to be the receptors by which innate immunity cells recognize invading microorganisms.

Among the 10 TLRs identified, TLR4 is the best characterized. Although TLR4 was identified as a receptor for the LPS of gram-negative bacteria, it also has been reported to be a receptor of endogenous ligands such as fibrinogen (23) and heat-shock protein 60 (24). Since levels of these endogenous ligands are known to be elevated in subjects with atherosclerosis (25), TLR4 is thought to play a crucial role in activating monocyte-macrophages in atherosclerosis.

Discussion

Monocyte-macrophages have been reported to play a key role in the pathogenesis of atherosclerosis and microorganisms such as Chlamydia pneumoniae and cytomegalovirus have been found to aggravate atherosclerosis by activating immune systems in the host. Further, toll-like receptors have been shown to be the receptors by which innate immunity cells recognize invading microorganisms.

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Fig. 4. Effect of LPS stimulation on TLR2 levels in SA and UA patients. Expression levels of TLR2 of LPS stimulated monocytes of patients with SA and UA are shown as normalized expression levels. The bar plots show medians in the 25th and 75th percentiles.

Fig. 5. Effect of LPS stimulation on TLR2 expression levels in SA patients.

Fig. 6. Effect of LPS stimulation on TLR2 expression levels in UA patients.

Fig. 7. Effect of LPS stimulation on TLR4 Levels in SA and UA Patients. TLR4 expression levels of LPS stimulated monocytes of patients with SA and UA are shown as normalized expression levels. The bar plots show medians in the 25th and 75th percentiles.
TLR2 was identified as a receptor for the peptidoglycan of gram-positive bacteria. Although no endogenous ligand of TLR2 has been found so far, it has been reported that, in endothelial cells (20) and macrophages (21), TLR2 is induced by TLR4 stimulation, thereby sensitizing cells to TLR2 ligands. As TLR4 and TLR2 have a common downstream signaling pathway, which leads to NF-κB activation, the cross-talk between TLR4 and TLR2 works as a positive feedback loop. Recently, Fan et al. have demonstrated that after intraperitoneal infection of wild type or TLR2 knockout mice by E. coli, the absence of TLR2 induction in the TLR2 knockout mice resulted in reduced expression levels of ICAM-1, and subsequently reduced migration of polymorph nuclear neutrophils into the lung (19). These findings suggest that TLR4 and TLR2 do not merely sense the specific ligands individually but rather act in concert to protect the host from infection (18).

Regarding the role of TLR4 and TLR2 in the pathogenesis of atherosclerosis, Edfeldt et al. reported that in human atherosclerotic plaques, TLR4 and TLR2 were expressed on macrophages invading atherosclerotic plaques and their immunoreactivities were co-localized with the nuclear translocation of NF-κB, a marker of the activation of this pathway, suggesting that TLR4 and TLR2 could play an important role in macrophage activation in such plaques (11).

In the present study, we demonstrated that expression levels of TLR4 and TLR2 on circulating monocytes are changed in CAD patients. We also found that the cross talk between TLR4 and TLR2 was differentially regulated in SA and UA patients. Thus the major findings of our study are as follows: 1) The expression levels of TLR4 on circulating monocytes were higher in SA and UA patients than in CNT patients and were not significantly different between the SA and UA patients. 2) The expression levels of TLR2 on circulating monocytes were higher in UA patients than in SA and CNT patients, and levels were not significantly different between the SA and CNT patients. 3) The response of TLR2 levels to LPS stimulation was differentially regulated in the SA and UA patients with up-regulation in SA patients and down-regulation in UA patients.

**Expression levels of TLR4 in SA and UA patients**

Our study showed that TLR4 levels were increased in both SA and UA patients. In human monocytes, increased surface expression of TLR4 by INF-γ has been reported to be associated with enhanced activation of the NF-κB pathway and subsequent production of inflammatory cytokines in response to LPS stimulation (26). Also, low serum levels of LPS have been detected in healthy subjects and shown to be an independent risk factor for atherosclerosis (27) and at low concentration ranges, LPS has been found to have significant proinflammatory effects on human blood vessels (28).

In subjects with carotid atherosclerosis, it was noted that serum levels of HSP 60, an endogenous ligand for TLR4, were also increased and there was a significant correlation between this and intima-media thickness (25). Another study found that serum levels of fibrinogen, another endogenous ligand for TLR4, were increased in patients with acute coronary syndrome (29). Taken together, these findings suggest that increased levels of TLR4 on circulating monocytes play an important role in the development of coronary plaques in CAD patients.

**Expression levels of TLR2 in UA patients**

In the present study, TLR2 levels were increased only in UA patients. Matsuguchi et al. (21) reported that in mouse macrophages, TLR4 messenger RNA was constitutively expressed and expression stayed at a constant level after stimulation with LPS or proinflammatory cytokines. On the other hand, TLR2 messenger RNA was expressed at low levels in the absence of stimulation but rose to high levels when cells were stimulated with either LPS or proinflammatory cytokines. Such LPS-mediated TLR2 messenger RNA induction was brought to an end by the blockade from NF-κB activation, suggesting that the NF-κB pathway plays an essential role in this regard.

Ritchie et al. (30) reported that the activation level of the NF-κB pathway, as indicated by the DNA binding of NF-κB in PBMCs, was much higher in UA patients than in SA patients. UA has also been reported to be associated with an inflammatory response, not only at the site of plaque rupture but also in the systemic circulation, as shown by increased plasma levels of CRP and proinflammatory cytokines (31) as well as by the activation of circulating monocytes (32, 33) and CD4-positive T lymphocytes (34). In these studies, the inflammatory marker levels were shown to be higher in UA than in SA patients. In our study, both activation of the NF-κB pathway in monocytes and increased levels of proinflammatory cytokines could explain the increased level of TLR2 expression on peripheral monocytes observed in the UA patients.

**Cross talk between TLR4 and TLR2 in SA and UA patients**

The cross talk between TLR4 and TLR2 was differentially regulated in the SA and UA patients. In the SA patients, TLR2 levels were increased by TLR4 stimulation with LPS, as previously reported for mouse macrophages and human endothelial cells. In contrast, in the UA patients, in which basal TLR2 levels were elevated, TLR2 levels were decreased by LPS stimulation. Given that the increased TLR2 levels on monocytes due to TLR4 stimulation works as a positive feedback loop, the decrease in TLR2 levels due to TLR4 stimulation in the UA patients
could have a protective role against excess stimuli. This response involving the TLR2 expression level is similar to that seen in activation levels of monocytes derived from UA patients due to LPS stimulation.

In this regard, Zalai et al. (33) measured the migration capacity and membrane fluidity of circulating monocytes as indices of their activation levels and showed that at baseline, monocyte activation levels were higher in UA than in SA patients. After LPS stimulation, the migration capacity of monocytes was increased in normal donors, but this was not the case for monocytes isolated from UA patients. The monocytes from the UA patients, however, could have already been activated and resistant to further activation. If the down-regulation of TLR2 levels after LPS stimulation has a protective role against excess stimuli, the elucidation of the mechanism of this regulation could lead to the identification of a new therapeutic option for modulating immunity activation levels in patients with CAD.

**Study limitations**

Our results suggested that the systemic inflammatory response in SA patients was associated with increased TLR4 expression levels on circulating monocytes, while the more prominent systemic inflammatory response in UA patients was associated with increased TLR2 expression levels. However, to confirm these observations, it would be necessary to investigate any changes in the expression pattern of these TLRs in the same patients. It would also be important to determine whether the induction of TLR2 expression is an event preceding plaque rupture or just a consequence of it. These issues should be clarified in future studies.

**Conclusions**

In the present study on surface expression levels of TLR2 and TLR4 on circulation monocytes in SA and UA patients, we showed that TLR4 levels were increased in both SA and UA patients, while TLR2 levels were increased only in UA patients. With LPS stimulation, the TLR2 levels were up-regulated in the SA patients but down-regulated in the UA patients.

**References**